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13. ABSTRACT (Maximum 200 Words) A growing body of evidence supports an oncogenic role for the ETS transcription factor ESX in breast cancer. ESX over-expression is detected in human breast cancer tissues and we have previously reported that the ESX-negative and nontransformed human breast cell line, MCF-12A, is transformed by stable ESX expression. In our current studies, we identify a unique 50-amino acid ESX domain, the SAR domain, that is sufficient to transform MCF-12A cells and whose transforming function is mediated by a novel cytoplasmic mechanism. Indeed, deletion of domains required for transcription factor function do not abrogate ESX-mediated MCF-12A cell transformation, whereas fusion of the SV40 large T antigen nuclear localization signal to either full-length ESX or to the ESX SAR domain alone blocked their transforming functions. Further, we find that ESX expression in transiently transfected cells is initially nuclear and mediates apoptosis. Together, our studies demonstrate two separate functions for the ESX transcription factor, apoptosis and transformation. Further, these two functions have distinct mechanisms: ESX-mediated apoptosis depends on nuclear ESX protein localization and appears to result from ESX function in transcription regulation. In contrast, ESX function in mammary epithelial cell transformation is mediated by the cytoplasmic function of its novel SAR domain.						
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Introduction

Members of the ETS transcription factor family play crucial roles in several different developmental processes, including differentiation and tissue formation (Graves and Petersen 1998; Oikawa and Yamada 2003). All ETS factors are characterized by a conserved winged helix-turn-helix DNA binding domain, the ETS domain, which mediates binding to ETS consensus sites in target genes (Kodandapani, Pio et al. 1996; Sharrocks 2001). ESX is a novel 47 kilodalton ETS factor that appears to mediate differentiation in some epithelial tissues (Oettgen, Alani et al. 1997; Neve, Chang et al. 1998). In addition to an ETS domain, the ESX protein includes a pointed domain, an A/T Hook domain, and a serine and aspartic acid-rich (SAR) domain (Chang, Scott et al. 1997). Aberrant expression of several ETS factors, including ESX, has been shown to promote oncogenesis. In humans, chromosomal translocations involving ETS factors are linked to Ewing's sarcoma and to chronic myelomonocytic leukemia (Delattre, Zucman et al. 1992; Golub, Barker et al. 1994; Dittmer and Nordheim 1998). Further, chickens infected with the E26 retrovirus, which encodes the *v-ETS* oncogene, develop hematopoietic malignancies (Leprince, Gegonne et al. 1983).

ESX over-expression appears to play an oncogenic role in human breast cancer. The ESX locus is amplified in roughly half of all early human breast tumors, and ESX mRNA over-expression is found in approximately 40% of human ductal carcinomas *in situ* (DCIS) (Chang, Scott et al. 1997; Tymms, Ng et al. 1997). Further, over-expression of the protooncogenic HER-2 receptor, which correlates with a highly aggressive breast cancer phenotype, is positively associated with ESX over-expression (Chang, Scott et al. 1997). ESX over-expression also imparts a motile and invasive phenotype to nontransformed human breast cells in culture [Schedin et al, in revision]. Further, an epithelial-to-mesenchymal morphological transition, which correlates strongly with transformation, is associated with stable ESX expression in nontransformed human breast cells [Schedin et al, in revision]. Taken together, these observations strongly suggest an oncogenic role for ESX over-expression in human breast cancer.

In this report, we have used subcellular protein localization and ESX coding sequence deletions to explore ESX function in breast cancer. We chose the MCF-12A cell line for many of our studies because this immortalized human breast cell line is not transformed and does not express endogenous ESX. Further, our previous studies indicate that stable ESX expression promotes anchorage independent growth, a phenotype consistent with transformation, in MCF-12A cells. Thus we used anchorage independent growth in soft agarose to correlate transformation with the subcellular localization of different stably expressed ESX deletion proteins. Our data reveal an entirely unanticipated mechanism of ESX action in breast cancer and suggest a novel mechanism whereby other ETS factors may participate transformation.

Body

As indicated in our original statement of work, we began our studies on the tumorigenic ESX protein with the assumption that the ESX protein functioned exclusively as a transcription factor in human mammary epithelial cells. This assumption was based on the presence of two homologous ETS transcription factor domains in the ESX protein: a pointed domain and an ETS domain (Chang, Scott et al. 1997). Previous in vitro studies had also demonstrated that exogenous ESX protein could bind to the ETS consensus site(s) in the promoters of several different human breast cancer-related genes. Further, binding of the ESX protein to these promoters was shown to activate downstream reporter gene transcription (Chang, Scott et al. 1997; Oettgen, Alani et al. 1997; Tymms, Ng et al. 1997; Choi, Yi et al. 1998; Eckel, Tentler et al. 2003). Given these data, we initially attempted to isolate endogenous ESX target DNAs, which, when dysregulated by ESX over-expression, might explain the mechanism of ESX action in breast cancer. However, we were consistently unable to isolate endogenous DNA targets of endogenous ESX protein from human breast cancer cells. We therefore began to question our assumption that the ESX protein functions as a transcription factor in breast cancer cells.

In order to test whether or not the ESX protein functioned endogenously as a transcription factor in human breast epithelial cells and to develop a new experimental approach for studying ESX function in breast cancer, we: (1) examined the subcellular localization of the endogenous ESX protein and of several GFP-fused ESX deletion mutants in human breast cells and (2) used colony formation in soft agarose to examine the transforming potential of each ESX deletion mutant, thus defining the ESX domain critical for ESX-induced breast cell transformation. Specifically, anti-ESX immunocytochemistry (IHC) revealed that endogenous ESX protein is cytoplasmically restricted in human breast cancer tissues. In contrast, a GFP-ESX fusion protein, expressed in MCF-12A cells, was either nuclearly localized or was expressed at levels below the limit of detection by fluorescence microscopy. Interestingly, nuclear GFP-ESX protein localization induced apoptosis in MCF-12A cells, apparently by activating a nuclear, pro-apoptotic gene expression program, whereas GFP-ESX negative MCF-12A transfectants were transformed. Further, deletion of domains required for ESX transcription factor function did not abrogate ESX-mediated MCF-12A cell transformation. Further, we found the ESX SAR domain, a 50 amino acid serine- and aspartic acid-rich domain that has no transactivating function and does not include a nuclear localization signal (NLS), is necessary and sufficient to transform MCF-12A cells. Finally, fusion of an exogenous NLS to either full-length ESX or to the ESX SAR domain alone blocked their transforming functions. Taken together, our data indicate that human mammary epithelial cell transformation can be induced by cytoplasmic ESX protein and that the ESX SAR domain mediates this transformation. This conclusion contrasts with the transcriptional model of ETS factor-mediated transformation and implies an entirely novel, cytoplasmic mechanism for ESX action in breast cancer.

Key Research Accomplishments and Conclusions

We have discovered that the ESX protein transforms human mammary epithelial cells via a novel, nonnuclear, nontranscriptional mechanism. This finding is supported by the following data:

- Endogenous ESX protein is restricted to the cell cytoplasm, both in the T-47D human breast cell line and in a sample of primary human ductal carcinoma *in situ* (DCIS).
- Deletion of domains required for ESX transcription factor function does not block ESX-mediated MCF-12A breast cell transformation. Indeed, nuclear localization of ESX protein blocks ESX-mediated MCF-12A cell transformation and can induce apoptosis in these cells.
- The 50 amino acid ESX SAR domain is necessary and sufficient to transform MCF-12A human breast cells. Further, nuclear localization of this domain abrogates its transforming function in MCF-12A human breast cells.

Together, these data not only reveal a novel, unexpected mechanism of ESX protein action in breast cancer but also represent an entirely new model for ETS factor function in tumorigenesis.

Reportable Outcomes

- This work was presented by JD Prescott (Principal Investigator) in an installment of the University of Colorado Health Sciences Center Program in Hormone-Related Malignancies seminar series, April, 2003.
- We are in the final stages of preparing a manuscript for publication that details the studies described here. We anticipate completion of this manuscript and submission to the journal Cancer Cell in September, 2003.

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